The Moloney Murine Sarcoma Virus *ts110* 5' Splice Site Signal Contributes to the Regulation of Splicing Efficiency and Thermosensitivity

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The 5' splice site signal (5'ss) in Moloney murine sarcoma virus *ts110* (MuSV*ts110*) RNA was found to participate in the regulation of its splicing phenotype. This 5'ss (CAG/GUAGGA) departs from the mammalian consensus (CAG/GURAGU) at positions +4 and +6, both of which base pair with U1 and U6 small nuclear RNAs during splicing. A doubling in splicing efficiency and near elimination of the splicing thermosensitivity characteristic of MuSV*ts110* were observed in 5'ss mutants containing a U at position +6 (termed 5'A6U), even in those in which U1-5'ss complementarity had been reduced. At the permissive temperature (28°C), the 5'A6U mutation increased the efficiency of the second splicing reaction, while at the nonpermissive temperature (39°C), both splicing reactions were positively affected.

Retroviral replication requires incomplete splicing of the genome length primary transcript. Unspliced RNA is translated into *gag* and *pol* gene products and used as genomic RNA for the packaging of virions, while spliced RNA is translated into *env* gene products. For several retroviral pre-mRNAs, it has been shown that suboptimal splicing signals near the 3' splice site signal (3'ss) mediate the balance between unspliced and spliced viral RNAs (2, 10, 11, 17, 18, 26, 29, 31, 39).

Interestingly, no regulatory role has been observed for the retroviral 5'ss. In two studies, it was found that a heterologous 5'ss could be substituted without consequence (11, 17). This apparent passivity might be unexpected, as the 5'ss makes several contacts with spliceosome components during splicing. For example, the 5'ss is specified by base pairing with U1 small nuclear RNA (snRNA) (8, 23), positions +1, +2, and +5 being the least tolerant of mutations, particularly in vitro (1). After the 5'ss is identified, U6 snRNA displaces U1 (9, 12, 14, 36) and base pairs with positions +5, +6, and, possibly, +2 (24, 25, 28, 36). While linked to the 5'ss, U6 snRNA forms intermolecular helices with U2 snRNA, itself paired with the branchpoint sequence (BPS). U2-to-U6 helix III may help align the 5'ss and the BPS, facilitating the first splicing reaction (32), and U2-to-U6 helices Ia and Ib are required for the second splicing reaction (16). Additionally, U5 snRNA contacts exonic sequences adjacent to the 5'ss and the 3'ss (21, 22, 28, 37), an interaction that may help align the exons for the second splicing reaction (28).

Considering the extensive involvement of the 5'ss in splicing reactions, we were prompted to ask whether it might help to regulate the unusual splicing phenotype of Moloney murine sarcoma virus *ts110* (MuSV*ts110*) RNA. MuSV*ts110* follows the general rules guiding retroviral RNA splicing but differs in that the splicing of its RNA is growth temperature regulated. At 33°C and below, MuSV*ts110* RNA splicing is 30 to 50% efficient but becomes progressively less efficient with rising

growth temperature until, at 39°C, splicing is virtually absent (3). We have previously shown that MuSV*ts110* splicing thermosensitivity is mediated in *cis* by a suboptimal BPS and polypyrimidine tract (6, 34), as well as by negatively acting sequences in the second exon (30). The wild-type (wt) MuSV*ts110* 5'ss (CAG/GUAGGA) is also suboptimal, departing from the mammalian consensus (CAG/GURAGU) at positions +4 and +6. Of these two mismatches, the position +4 mismatch seemed unlikely to affect splicing, as a G-U bond would still be permitted with U1 snRNA. However, position +6 contacts both U1 and U6 snRNAs during splicing, and hence, a mismatch at this position seemed very likely to decrease the efficiency of splicing.

We focused on position +6 in the MuSV*ts110* 5'ss, introducing "megaprimer"-based mutations predicted to alter base pairing with U1 and/or U6 snRNA (Fig. 1). The effect of these mutations on splicing was assessed by S1 analysis after transfection (7) into NIH 3T3 cells, using a probe containing a plasmid DNA extension which excluded reannealed probe from consideration in the assay (6, 19, 20, 34). Splicing efficiency was quantitated with a Betagen Betascope.

Position +6 was first mutated to U (CAG/GUAGGAA to CAG/GUAGGUA, termed 5'A6U) (Fig. 2C). This mutation had distinct effects on splicing reactions at 28 and 39°C. For example, wt MuSV*ts110* RNA was spliced at efficiencies of approximately 30 and <1% at 28 and 39°C, respectively (Fig. 2A, lanes 1 and 2, and Table 1). However, when the fraction of RNA that was cleaved at the 5'ss and/or fully spliced (termed the C/S pool) was estimated by using a 5'ss probe (pBAM-E1; Fig. 2B), 53% of the wt MuSV*ts110* RNA was found in this pool at 28°C, while essentially no wt C/S RNA was observed at 39°C (Fig. 2B, lanes 1 and 2, and Table 1). Hence, it was apparent that an additional 20 to 25% of the wt viral RNA had completed step 1 but not step 2 of splicing and that no splicing reactions had been completed at 39°C. In the 5'A6U mutant, splicing efficiency was doubled (62%) at 28°C and increased to 34% at 39°C (Fig. 2A, lanes 3 and 4, and Table 1). Interestingly, however, the C/S and fully spliced RNA pools in the 5'A6U mutant were approximately equal at both 28 and 39°C, respectively, arguing that there was no pool of cleaved but unspliced 5'A6U intermediates at either growth temperature

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FIG. 1. MuSVts110 5'ss mutants. (A) Complementarity of the MuSVts110 5'ss with motifs in U1 and U6 snRNAs. The MuSVts110 5'ss (5'-CAG/GUAGGAAC-3'; MuSVts110 nucleotides 2043 to 2053) is shown base paired with the conserved 3'-GAGACA-5' motif in U6 snRNA (U6 snRNA nucleotides 41 to 46) and with the 5' end (3'-GUCCAUA-5') of U1 snRNA (U1 nucleotides 2 to 12). (B) MuSVts110 5'ss mutants generated for this study.

(Fig. 2A and B, lanes 3 and 4, and Table 1). Considered together, these data indicated that at 28°C, wt MuSVts110 RNA splicing was partially restricted after step 1 of splicing. The 5'A6U mutation eliminated this restriction. At 39°C, however, the 5'A6U mutation had the dual effect of relieving the virtually complete blockade normally observed prior to step 1 at 39°C, as well as the partial restriction after step 2 seen at 28°C. As both U1 and U6 are involved in step 1 of splicing and U6 is involved in the second step, these data did not allow a distinction between U1 and U6 effects.

Although the data strongly suggested that the balance between unspliced and spliced MuSVts110 RNAs, as well as their characteristic splicing thermosensitivity, is sensitive to the complementarity of position +6 to U1 and/or U6 snRNA, it was possible that the observed effect was nucleotide specific and unrelated to 5'ss-snRNA complementarity. As a test, we generated two forms of pseudo-wt 5'ss (5'A6C and 5'A6G) (Fig. 2C), in both of which position +6 would be incapable of base pairing with either U1 or U6 snRNA. Splicing of these mutant RNAs was, like that of wt MuSVts110, inefficient (28 to 36%) and thermosensitive (Fig. 2A, lanes 5 to 8), a strong argument that the effect of the 5'A6U mutation stemmed from an enhancement of 5'ss-U6 or -U1 snRNA complementarity.

We next asked if the enhanced splicing seen in the 5'A6U mutant was specific for position +6 or if other mutations increasing 5'ss-U1 or -U6 complementarity could enhance splicing efficiency. As a test, we placed a U at position +4 (CAG/GUAGGAA to CAG/GUAUGAA, termed 5'G4U), allowing a new U6 snRNA-5'ss base pair at +4 while leaving position +6 mismatched and, at the same time, eliminating the position +4 G-U bond to U1 snRNA (Fig. 3B). In a second mutant, we placed a U at positions +4 and +6 (CAG/GUAGGAA to CAG/GUAUGUA, termed 5'G4U/A6U), permitting a total of four base pairs with U6 and nine base pairs with U1 (Fig. 3B). S1 analysis indicated that the splicing of 5'G4U RNA was little different from that of wt MuSVts110, although a slight increase in splicing at 39°C was noted (Fig. 3A, lanes 3 and 4). The splicing pattern of 5'G4U/A6U RNA was very similar to that of RNA containing only the 5'A6U mutation (Fig. 3A, lanes 5, 6). Considered together, these observations suggested that position +6-snRNA complementarity is critical. Moreover, as the position +4-U1 mismatch neither had a deleterious effect on splicing nor diminished the enhancement of splicing produced by the 5'A6U mutation, these experiments suggested that the

FIG. 2. Splicing efficiency and thermosensitivity as a function of the identity of the nucleotide (nt) at position +6 in the MuSVts110 5'ss. 5'ss variants containing either A (wt), U (5'A6U), C (5'A6C), or G (5'A6G) at position +6 were constructed, transfected, and subjected to S1 analysis for exon ligation. U, unspliced RNA; S, spliced RNA. (A) Exon ligation in position +6 mutants. Lanes: 1 and 2, wt MuSVts110 RNA at 28 and 39°C, respectively; 3 and 4, 5'A6U RNA at 28 and 39°C, respectively; 5 and 6, 5'A6C RNA at 28 and 39°C, respectively; 7 and 8, 5'A6G RNA at 28 and 39°C, respectively. (B) 5'ss cleavage-exon ligation in wt and 5'A6U RNAs. C/S, cleaved-spliced RNA. Lanes: 1 and 2, wt MuSVts110 RNAs from cells grown at 28 and 39°C, respectively; 3 and 4, 5'A6U RNA from cells grown at 28 and 39°C, respectively; M and P, marker (pBR322 DNA digested with *Msp*I) and probe alone, respectively. (C) Complementarity of the various 5'ss variants to U1 and U6 snRNAs.

effect of the 5'A6U mutation on MuSVts110 RNA splicing might be explained by improved U6-5'ss complementarity.

Mutation of both positions +3 and +4 to U (CAG/GUAGGAA to CAG/GUUUGA, termed 5'A3U/G4U) (Fig. 4B) emphasized the significance of position +6 in the MuSVts110

TABLE 1. Comparison of levels of spliced wt MuSVts110 and 5'A6U RNAs with the total amount of RNA in which 5'ss cleavage and splicing were completed

Temp (°C)	% Spliced, % cleaved and spliced	
	wt MuSVts110	5'A6U
28	32, 53	62, 58
39	<1, <1	34, 35

FIG. 3. Splicing and complementarity of nucleotides +4 and +6 in the MuSVts110 5'ss. 5'ss variants containing U at either position +4 (5'G4U) or both positions +4 and +6 (5'G4U/A6U) were constructed, transfected, and subjected to S1 analysis for exon ligation as described in Materials and Methods and the legend to Fig. 2. (A) Exon ligation in position +4 and position +4 and +6 5'ss mutants. Lanes: 1 and 2, wt MuSVts110 RNA at 28 and 39°C, respectively; 3 and 4, 5'G4U RNA at 28 and 39°C, respectively; 5 and 6, 5'G4U/A6U RNA at 28 and 39°C, respectively. (B) Complementarity of 5'G4U and 5'G4U/A6U 5'ss mutants to U1 and U6 snRNAs. U, unspliced RNA; S, spliced RNA.

5'ss. This double mutation created a two-base internal mismatch with U1 snRNA, predictably eliminating splicing at both 28 and 39°C (Fig. 4A, lanes 3 and 4). Interestingly, placement of a U at position +6 in the 5'A3U/G4U 5'ss (CAG/GUUU GU, termed 5'A3U/G4U/A6U) allowed recovery of the wt splicing level (30%) at 28°C (Fig. 4A, lane 5) and a degree of splicing at 39°C (11%) substantially higher than expected for wt splicing (Fig. 4A, lane 6). These results suggested that the additional A6U mutation had both restored 5'ss recognition, almost certainly by improving 5'ss complementarity to U1 and, through an additional effect mediated by either U1 or U6 complementarity at position +6, enhanced the efficiency of step 2 of splicing in a fashion reminiscent of the effect of the 5'A6U mutation alone.

S1 analysis with the 5'ss probe was carried out to determine if any of the mutations other than 5'A6U affected steps 1 and 2 of splicing differently. This analysis was done as already described (Fig. 2), with one difference. In the experiment whose results are shown in Fig. 2, we used a wt probe with mutant RNA and it was possible that digestion at the probe-5'ss mismatch might exaggerate the size of the C/S RNA pool. To sidestep this potential problem, we developed gel conditions capable of resolving authentic and spurious protection fragments. For example, in 5'A3U/G4U/A6U RNA analyzed with the pBAM-E1 probe, we expected authentic S1 protection fragments of 341 and 235 nucleotides and, possibly, a 237-nucleotide spurious fragment (Fig. 5C). As shown in Fig. 5B, a 235- and 237-nucleotide doublet was resolved. Quantitation indicated that about half of the 5'A3U/G4U/A6U RNA was in the C/S pool, a result in general agreement with the exon ligation data (30% spliced; Fig. 4A). By using this assay, we

FIG. 4. Splicing in MuSVts110 mutants containing double and triple mutations in the 5'ss. 5'ss variants containing U at either positions +3 and +4 (5'A3U/G4U) or +3, +4, and +6 (5'A3U/G4U/A6U) were constructed, transfected, and subjected to S1 analysis for exon ligation as described in Materials and Methods and the legend to Fig. 2. (A) Exon ligation in position +3 and +4 and position +3, +4, and +6 5'ss mutants. Lanes: 1 and 2, wt MuSVts110 RNA at 28 and 39°C, respectively; 3 and 4, 5'A3U/G4U RNA at 28 and 39°C, respectively; 5 and 6, 5'A3U/G4U/A6U RNA at 28 and 39°C, respectively. (B) Complementarity of 5'A3U/G4U and 5'A3U/G4U/A6U 5'ss mutants to U1 and U6 snRNAs. U, unspliced RNA; S, spliced RNA.

tested all of the 5'ss mutants except 5'A6C and 5'A6G. The results were as follows: 5'A6U RNA protected the 341- and 253-nucleotide S1 fragments at 28 and 39°C; no 237-nucleotide fragment was observed, indicating that a single mismatch did not result in spurious digestion under these conditions (Fig. 5A, lanes 3 and 4). In the wt-like 5'G4U RNA, 5'ss cleavage was, as expected, thermosensitive (Fig. 5A, lanes 5 and 6). In the 5'A6U-like G4U/A6U double mutant, 5'ss cleavage was, as expected, essentially indistinguishable from that of 5'A6U (Fig. 5A, lanes 10 and 11). As anticipated, the splicing-negative 5'A3U/G4U RNA protected only the 237-nucleotide probe fragment (Fig. 5A, lanes 7 and 8). Finally, in 5'A3U/G4U/A6U RNA, in which the effect of the A3U/G4U mutation was reversed, both the authentic 235-nucleotide and spurious 237-nucleotide protection fragments were found in approximately equal amounts (Fig. 5A, lanes 12 and 13; also, Fig. 5B).

These experiments show that the 5' splice site signal can participate in the regulation of MuSVts110 splicing and allow some conclusions concerning the nature of this regulation. The inefficient, thermosensitive wt MuSVts110 splicing phenotype tolerates nucleotides at position +6 which are not complementary to U1 and/or U6 snRNA and also tolerates an additional position +4 mismatch with U1. However, when position +6 in the MuSVts110 5'ss was complementary to U1 and U6, even in 5'ss mutants with a two-base internal mismatch with U1, splicing efficiency was increased as much as twofold and splicing

FIG. 5. 5'ss cleavage-exon ligation in MuSVts110 5'ss mutants. The degree of 5'ss cleavage and exon ligation in the panel of 5'ss mutants was measured by S1 analysis with the pBAM-E1 probe. Protected probe fragments were analyzed on a 20% polyacrylamide gel containing 8 M urea. U*, spuriously digested unspliced RNA. (A) 5'ss cleavage-exon ligation. Lanes: 2, wt MuSVts110 RNA at 28°C; 3 and 4, 5'A6U RNA at 28 and 39°C, respectively; 5 and 6, 5'G4U RNA at 28 and 39°C, respectively; 7 and 8, 5'A3U/G4U at 28 and 39°C, respectively; 9, wt MuSVts110 RNA at 28°C; 10 and 11, 5'G4U/A6U RNA at 28 and 39°C, respectively; 12 and 13, 5'A3U/G4U/A6U RNA at 28 and 39°C, respectively; 1, M (marker [pBR322 digested with *Msp*I]). (B) S1 analysis of 5'G4U/A6U RNA at 28°C. (C) S1 nuclease protection pattern of pBAM-E1-RNA hybrids.

thermosensitivity was eliminated or greatly reduced. The increase in splicing efficiency at low temperature appeared to localize to step 2 of splicing, suggesting that U1 was not involved since it is not required at this step (5, 33). Interestingly, the substantial relaxation of splicing thermosensitivity afforded by the same mutation proceeded by a distinct, although presumably related, mechanism. The wt MuSVts110 RNA completed no splicing reactions at 39°C, but in RNA containing the 5'A6U mutation, both steps 1 and 2 occurred with equivalent efficiencies at 39°C.

The known dispensability of U1 for step 2 in splicing suggested a model invoking position +6 complementarity to U6 snRNA, at least at 28°C. However, within the U6 ACAGAG motif that interacts with the 5'ss, mutation of the first four nucleotides (underlined) affects step 1 of splicing, while mutation of the last two nucleotides (boldface) affects step 2 (reviewed in reference 35). As the 5'A6U mutation in MuSVts110 targets base pairing of the 5'ss with the first nucleotide in the U6 motif, an effect on step 1 would be expected. The anticipated result was observed at 39°C, at which the 5'A6U mutation caused both steps 1 and 2 to accelerate in concert, but at 28°C, only step 2 was enhanced. On the basis of a large body of previous work in other systems, it seems virtually certain that the ability of the 5'A6U mutation to allow step 1 to proceed at 39°C was due to improved complementarity of the 5'ss to U1 and/or U6 snRNA which facilitated 5'ss recognition and the alignment of the 5' end of the intron with the BPS (27, 32). To explain the effect of the 5'A6U mutation on step 2 of splicing, we speculate that it may have helped to stabilize intermolecu-

lar helices between U6 and U2 that are required for step 2 of splicing. The 5'A6U mutation, by providing a critical base pair with U6, would be expected to improve its tethering to the 5'ss. During step 1 of splicing, U6, base paired to the 5'ss, is aligned with the BPS bound to U2, allowing formation of the U2-to-U6 helices required for step 2. Binding of U2 to the BPS is facilitated by U2AF binding to the polypyrimidine tract (13, 38). Interestingly, in another pre-mRNA, a 5'ss mutation (G to U at position +6) equivalent to the MuSVts110 5'A6U mutation has been reported to facilitate U1 small nuclear ribonucleoprotein particle-mediated binding of U2AF65 to the polypyrimidine tract (4). We view MuSVts110 pre-mRNA as an excellent candidate for the enhancement of U2 binding to the BPS mediated by improved U1-5'ss complementarity, as both of these splicing signals are suboptimal in MuSVts110. Provision of a consensus BPS and/or polypyrimidine tract containing an optimal U2AF binding site greatly increases MuSVts110 splicing efficiency and substantially lessens splicing thermosensitivity (15, 34). Hence, we speculate that improved binding of U1 to the 5'A6U 5'ss ultimately results in enhanced U2 binding to the weak wt BPS and that this effect should persist even after U1 is displaced from the active spliceosome. At the same time, improved tethering of U6 to the 5'A6U 5'ss once U1 is displaced may also promote formation of the U2-to-U6 intermolecular helices required for the second splicing reaction.

Previously, we reported that alterations in the MuSVts110 BPS and polypyrimidine tract predicted to enhance their interactions with spliceosome components can dramatically increase splicing efficiency and, at the same time, lessen or even eliminate splicing thermosensitivity (6, 34). The extension of these findings to the 5'ss reinforces our earlier suggestion that MuSVts110 splicing thermosensitivity stems from a web of weak, interdependent splicing signal-spliceosome component interactions which become increasingly unstable at higher temperatures. The network nature of these interactions is underscored by our observations that individual optimization of any of these splicing signals dramatically improves splicing efficiency (in the order BPS > polypyrimidine tract > 5'ss).

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